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Exposure of Chinese hamster ovary (CHO) and African green monkey kidney (VERO) cells to T-2 mycotoxin resulted in several morphological changes which appeared to be directly related to inhibition of protein synthesis, the basic in vitro mechanism of action of the toxin. These changes, wihich occurred in both cell types, included dissociation of polysomes and mitochondrial cristae alterations. In addition, CHO cells displayed membrane bleb formations which were either a result of protein synthesis inhibition or a specific early pathological response.

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Bleb formations were not observed in VERO cells. Similar morphological changes were found in both cell types exposed to established inhibitors of protein synthesis, puromycin or anisomycin.

Morphological Changes in CHO and VERO Cells Treated with T-2 Mycotoxin. Correlation with Inhibition of Protein Synthesis

Lynn R. Trusal

Pathology Division

U.S. Army Medical Research Institute of Infectious Diseases

Ft. Detrick

Frederick, MD 21701

Running Title: T-2 Toxin Effects on Cultured Cells

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Send correspondence and proofs to:

Dr. Lynn R. Trusal
Pathology Division
U.S. Army Medical Research Institute of Infectious Diseases
Ft. Detrick
Frederick, MD 21701

Synopsis

Exposure of Chinese hamster ovary (CHO) and African green monkey kidney (VERO) cells to T-2 mycotoxin resulted in several morphological changes which appeared to be directly related to inhibition of protein synthesis, the basic *in vitro* mechanism of action of the toxin. These changes, which occurred in both cell types, included disassociation of polysomes and mitochondrial cristae alterations. In addition, CHO cells displayed membrane bleb formations which were either a result of protein synthesis inhibition or a specific early pathological response. Bleb formations were not observed in VERO cells. Similar morphological changes, were found in both cell types exposed to established inhibitors of protein synthesis; puromycin or anisomycin.

Introduction

T-2 toxin [3α-hydroxy-4β,15-diacetoxy-8-α(3-methylbutyryloxy)-12,13-epoxytrichothec-9-ene], is a potent mycotoxin produced by the Fusarium species[1]. T-2, along with other trichothecene mycotoxins, is one of the most potent inhibitors of eukaryotic protein synthesis[2]. The in vitro mechanism of action of T-2 toxin involves its ability to bind to the 60S subunit of ribosomes[3,4], thereby blocking initiation of protein synthesis[5]. In addition, T-2 toxin has been shown to cause disaggregation of polyribosomes[6,7].

Although much has been written about the structure and mode of action of T-2 toxin, little information on its morphological and ultrastructural effects has been published. The aim of the current study is to correlate the inhibition of protein synthesis by T-2 with morphological observations in CHO and VERO cell lines. In addition, observed changes will be compared with other inhibitors of protein synthesis.

Experimental

Cultured cell lines

Established cell lines of Chinese hamster ovary (CHO) and African green monkey kidney cells (VERO) (Amer. Type Culture Collection, Rockville, MD) were used in each experiment. Cells were maintained in Earles minimal essential media (EMEM)(GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin and 2.5 µg/ml fungizone. Cultures were kept in a 37°C, 5% CO_2 incubator.

Toxin

T-2 toxin (Calbiochem-Behring, San Diego, CA) was maintained at a stock concentration of 5 mg/ml in ethanc The purity of the toxin was determined to be 99% by gas liquid chromatography and thin layer chromatography. Just prior to use, T-2 toxin was diluted to the desired concentration in tissue culture media. Cells used for either morphological examination or inhibition of protein synthesis were exposed to either 0.01 µg/ml or 1.0 µg/ml T-2 toxin for 1 or 12 hrs. Other experimental points included 1 or 12 hrs toxin exposure followed by removal of toxin and incubation for 12 hrs in medium only.

Inhibition of Protein Synthesis Assay

CHO and VERO cells were seeded into 24-well tissue culture dishes (Costar, Cambridge, MA) at a density of $8x10^4$ cells/well, and incubated in a 37°C warm box. Medium H-199 (GIBCO, Grand Island, NY) was supplemented with 10% fetal calf serum, 25 mM HEPES buffer, 100 units/ml penicillin, and 100 µg/ml streptomycin. The following day, the medium was removed and fresh medium was added. Medium contained either no toxin (controls) or T-2 toxin at a concentration of 0.01 or 1.0 µg/ml. Toxin was added in a reverse time sequence (i.e. longest exposure first) so that all wells could be processed simultaneously. At the appropriate time period, toxin was removed and the wells rinsed with Hanks balanced salt solution (HBSS). Tritiated [^{3}H] leucine (144Ci/mM) (in culture medium), at a concentration of 2 uCi/ml, was added to each well, and cultures were incubated for 30 min (37°C). Following leucine incorporation, the cells were washed with HBSS and lysed with 0.1N NaOH. Half of the lysed cell suspension was used for determination of total proteins,[8] and half was used for protein synthesis determinations[9]. Protein synthesis

values were normalized for total protein content in each well and represented the mean of 4 samples per time point. Final values represent percent inhibition of protein synthesis values in treated cells versus controls.

Morphologic Examination

Cultures for morphology and ultrastructure studies were seeded into Leighton tubes (Costar, Cambridge, MA) at a density of 5 x 10^5 cells/tube in complete EMEM. Each tube contained a plastic (polymethylpentene) coverslip on which the cells formed confluent monolayers. The portion of the coverslip to be used for scanning (SEM) (AMRay, Bedford, MA) and transmission electron microscopy (TEM) (JOEL USA, Medford, MA) was processed as previously described[10]. After fixation, a portion of the coverslip was excised and examined either by phase contrast or stained for light microscopy (LM) examination. In this manner, the same monolayer (i.e. coverslip) was processed for examination by phase contrast, bright field, and SEM and TEM examination.

Antibiotic Studies

Both puromycin and anisomycin were obtained from the Sigma Chemical Company, St. Louis, MO. Puromycin was dissolved in culture medium and added to confluent CHO or VERO cell cultures (Leighton tubes) at a concentration of 35 µg/ml or 100 µg/ml, respectively. Anisomycin was dissolved in ethanol and diluted in culture medium to a concentration of 0.06 µg/ml for CHO cultures and 0.01 µg/ml for VERO cultures. Doses of both puromycin and anisomycin were chosen to approximate the percent inhibition of protein synthesis (% IPS) caused by 1.0 µg/ml of T-2 for 1 hr in CHO and VERO cells (Dr. John Middlebrook, personal communication). This was done to preclude morphological

changes which might be dosage-dependent. Identical time points were used, and coverslip cultures of each cell type were processed for LM, SEM, and TEM examination.

Results

Table 1 illustrates inhibition of protein synthesis by T-2 toxin in CHO and VERO cells. In CHO cells, protein synthesis was approximately 80% of controls after 12 hrs exposure to 0.01 μg/ml T-2 toxin. Twelve hrs after washing out the toxin there was a complete recovery of protein synthesis. At the same concentration of T-2 toxin, VERO cells appeared to be more sensitive, with protein synthesis 23% of controls after 12 hrs. When toxin was removed, recovery was nearly complete.

At 1.0 μg/ml, protein synthesis dropped to 16% of controls by 12 hrs in both cell types. Removal of toxin and replacement by culture medium resulted in no appreciable recovery of the protein synthesis capability of either cell type. Compared to the lower toxin dose, there was no appreciable difference between CHO and VERO cells in their response to inhibition of protein synthesis. At the higher toxin dose, a longer toxin exposure (12 hrs versus 1 hr) did not further decrease protein synthesis.

Light microscope examination of control CHO (Fig. 1A) and VERO (Fig. 1B) cultures revealed few differences. VERO cells occupied a larger surface area on the substrate than CHO and were more epithelial in appearance. When both cultures were exposed to T-2 toxin under identical conditions, morphological changes visible in the light microscope were detectable in CHO cells but not in VERO. After exposure to 0.01 μg/ml toxin for 12 hrs, the major visible change was an increasing number of CHO cells with surface blebs. The percentage of cells displaying such blebs increased with increasing toxin

concentration and time of exposure. After a dose of 1.0μg/ml T-2 for 12 hrs, followed by a toxin recovery period (12 hrs), the CHO cells did not revert back to control morphology (Fig. 2). In contrast, VERO cultures did not demonstrate any change in LM morphology at any combination of toxin concentrations or times of exposure.

SEM examination of the plasma membrane of these cells visualized the morphological changes in greater detail. Fig. 3A (1.0 μg/ml-12 hrs) shows a significant number of CHO cells with extensive surface membrane blebs not found in control cultures. Neither control nor experimental (Fig. 3B, 1.0 μg/ml 12 hrs) VERO cells displayed surface blebs.

When cultures of both cell types were exposed to anisomycin or puromycin, results were similar. Examination by LM and SEM revealed that CHO cells exposed to either anisomycin (0.06 μg/ml) or puromycin (35 μg/ml) developed membrane blebs while VERO cells did not. Fig. 4 illustrates a sample CHO culture exposed to anisomycin (0.06 μg/ml, 12 hrs).

Sample TEM micrographs of CHO and VERO controls are shown in Figs. 5A and 5B, respectively. Both control cell types displayed generally similar morphology. Each cell contained nuclei with prominent nucleoli and a relative homogeneous chromatin distribution. The cytoplasm contained microtubules, thin filaments, golgi, ribosomes, numerous mitochrondria and limited rough endoplasmic reticulum. Differences between the two cell types were noted largely at the mitochondrial and ribosomal levels. CHO cell mitochondria presented largely oval profiles with fine cristae and a homogenous matrix. VERO cell mitochondria tended to display longitudinal profiles and denser matrices. In both cell types, rough endoplasmic reticulum was limited and polyribosome formation differed in appearance. CHO cell polysomes usually appear as evenly distributed rosette formations. In addition to rosette

shaped polysomes, VERO cells also contain numerous spiral formations (Fig. 5B, box) not seen in CHO cultures.

Ultrastructural examination of CHO cells exposed to T-2 toxin (1.0 μg/ml, 12 hrs) revealed that the nucleus, nuclear membrane, chromatin, microtubules, and thin filaments appeared unaltered. However, some alterations were observed at this 12 hr time period. In CHO cells, membrane blebs appeared as non-fluid filled structures containing ground substance, ribosomes, and an occassional mitochondrion (Fig. 6) The other noticable alteration was a generalized dissaggregation of polysomes. This was first observed at a dose of 0.01 μg/ml of T-2 for 12 hrs, although not all cells exhibited it. By 12 hrs at a dose of 1.0 μg/ml, ribosomes appeared as monosomes scattered homogeneously throughout the cytoplasm (Fig. 7A, CHO).

Although VERO cells did not exhibit surface membrane bleb formation, there was a dissaggregation of polysomes (Fig. 7B). No spiral or rosette polysomes were found, and monosomes were found homogeneously distributed in the cytoplasm. Even after a 12 hr recovery period for both cell types, ribosomes appeared predominatly as monosomes.

Like T-2 toxin, both puromycin and anisomycin produced similar morphological changes at corresponding concentration/time points. In CHO cells, surface blebs were evident after 12 hrs exposure to either drug (Fig. 8). In both cell types, polysomes were disaggregated into monosomes after 1 hr exposure to either drug; transformation apppeared largely complete by 12 hrs. (Fig. 8, CHO).

In both toxin and drug-treated VERO cells, and to a lesser degree, CHO cells, mitochondria displayed an increase in matrical density, ballooning of the intracristal space, and changes in cristae orientation (Fig. 9). In addition, VERO cultures contained occassional mitochondria with condensed

configurations (Fig. 9, inset). Despite the other changes, there was no outer compartment swelling of the mitochondria, and the outer membrane remained intact.

Discussion

The ability of T-2 mycotoxin to inhibit protein synthesis in eukaroytic systems has been well documented[2], but a review of the literature has revealed limited information on its morphological or ultrastructural effects *in vitro*[11].

We chose to examine the morphological effects of T-2 toxin on two cultured cell lines used in our laboratory to study uptake, internalization and metabolism of T-2 in vitro. In addition, we correlated morphological observations with inhibition of protein synthesis, its major action *in vitro*. Inhibition of protein synthesis was reversible at the low dose (0.01 µg/ml), whereas at the higher dose (1.0 µg/ml), there was little recovery of protein synthesis after removal of the toxin (Table 1).

It was clearly evident that T-2 toxin caused marked changes in the surface morphology of CHO but not VERO cells, as evidenced by both LM and SEM. The appearance of blebs on the cell surface was reported to be a pathological response to various insults, such as acute ischemia or inhibition of glycolysis and cellular respiration[12]. Whereas blebs caused by these insults tend to be of low viscosity,[12], the blebs on the CHO cells were of the same internal density as the cellular ground substance and did not appear to be the result of water loss.

Because T-2 toxin produced plasma membrane changes in CHO but not VERO cells, we examined its relationship to inhibition of protein synthesis. Two antibiotics which inhibited protein synthesis by different mechanisms were chosen. Anisomycin, like T-2, is believed to interfere with initiation of

protein synthesis by binding to the 60S ribosomal subunit and altering peptidyl transferase activity[13,14]. Puromycin, on the other hand, interferes with normal peptide bond formation and causes premature release of protein chains[15].

The mechanism underlying T-2 induced bleb formation in CHO but not VERO cells is unclear, although it appears that bleb formation is not a specific alteration induced only by T-2. Since puromycin and anisomycin induced the same pattern, it is tempting to suggest that CHO cells respond to inhibition of protein synthesis by forming blebs, while VERO cells do not. It is also possible that such formation is unrelated to inhibition of protein synthesis and in CHO as with HeLa cells,[12] blebing is one of the earliest pathological responses to various insults. In addition, we have consistently found that VERO cells take up more T-2 toxin on a molecules/cell basis (manuscript in preparation) and also exhibit greater inhibition of protein synthesis than CHO cells under identical conditions (Table 1). This observation supports the conclusion that formation of blebs in CHO cells is not the direct result of inhibition of protein synthesis caused by T-2.

While examination of surface morphology revealed CHO versus VERO differences, examination of internal ultrastructure demonstrated mostly similarities. The nucleus remained largely unchanged in both CHO and VERO cells in response to T-2, puromycin, or anisomycin. The nuclear chromatin distribution, nucleolar appearance, and nuclear pores were similar to control cultures of both cell types. The only observed change was an increase in the irregularity of nuclear shape in CHO cells exposed to T-2 or anisomycin. Irregular nuclear envelopes have been reported in human fibroblasts exposed to T-2[11]. This report also states that T-2 toxin causes margination of chromatin[11]. This was not evident in our cells to an extent greater than controls.

The greatest interior changes were noted in the cytoplasmic compartment of both cell types. Dissociation of polyribosomes was clearly the predominant event. In VERO cells, spiral polysomes and endoplasmic reticulum bound ribosomes were transformed to monosomes; while in CHO cells, rosette and endoplasmic reticulum-bound polysomes also disappeared. The concomitant dissociation of polysomes in puromycin-and anisomycin-treated cells indicates that, regardless of the specific mechanism of inhibition of protein synthesis (i.e. initiation versus termination), polysomal disaggregation was a common occurrence.

The other cytoplasmic organelle which appeared altered by toxin or drug exposure was the mitochondrion. The increase noted in the intracristal space (i.e. ballooning) has not been reported in other cell systems exposed to T-2[11] or other mycotoxins[10]. It is interesting to note that a mutant line of Chinese hamster lung fibroblasts defective in mitochondrial protein synthesis displays mitochondria with ballooned or tubular cristae[17]. Furthermore, chloramphenicol, which inhibits prokaryotic and mitochondrial ribosomal protein synthesis, has been shown to cause a loss of cristae orientation[17,18]. Therefore, the ultrastructural effects of T-2 mycotoxin on mitochondria may reflect inhibition of mitochondrial protein synthesis in these cultured cells. Support for this conclusion comes from studies with isolated rat liver mitochondria which displayed a 70% inhibition of mitochondrial protein synthesis in response to T-2 (Dr. Judith Pace, manuscript in preparation). In addition, recent work has shown that T-2 inhibits hepatic mitochondrial respiration, which may explain the occurrence of occassional mitochrondria with condensed cristae configurations[12,19].

In summary, when CHO and VERO cells are exposed to various doses of T-2 mycotoxin, several prominent morphological alterations occur. These include surface bleb formation in CHO cells and dissociation of polysomes and

mitochondrial cristae changes in both cell types. While mitochondrial and ribosomal changes appear directly related to inhibition of protein synthesis, bleb formation does not. Further work is needed to elucidate the exact mechanism of ultrastructural changes in cultured cells exposed to T-2 or other mycotoxins.

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Disclaimer

The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Table 1

Inhibition of Protein Synthesis by T-2 Mycotoxin in CHO and VERO Cells

Cell Type		T-2 Toxin Dose (μg/ml) 0.01	1.0
CHO		% Protein Synthesis *,†	
Exposure time	Recovery time		
1 (hr)		91 ± 4.8	19 ± 12.8
1 (hr)	12 (hrs)	99 ± 4.4	28 ± 6.1
12 (hrs)		81 ± 9.1	16 ± 5.6
12 (hrs)	12 (hrs)	100 ± 9.5	35 ± 8.5
VERO			
Exposure time	Recovery time		
1 (hr)		34 ± 5.2	15 ± 2.9
1 (hr)	12 (hrs)	85 ± 23.4	19 ± 2.3
12 (hrs)		23 ± 3.1	15 ± 2.9
12 (hrs)	12 (hrs)	100 ± 25.9	23 ± 1.8

*= Data equal mean of 5 experiments

†= Values are expressed as percent of control protein synthesis where controls equal 100%.

Figure Legends

Figure 1. Light micrograph of control cell cultures (A) CHO, (B) VERO. Bar = 10μ.

Figure 2. Light micrograph of CHO cells exposed to 1.0 ug/ml T-2 toxin for 12 hrs followed by medium for 12 hrs. Note blebs on most cells (arrows). Bar = 10μ.

Figure 3. SEM micrographs of cell cultures exposed to T-2 toxin (A) CHO cells, 1.0 μg/ml T-2, 12 hrs. Note surface membrane blebs (arrows). (B) VERO cells, 1.0 μg/ml T-2, 12 hrs Bar = 10μ.

Figure 4. SEM micrograph of CHO cells exposed to 0.06 μg/ml of anisomycin for 12 hrs. Note bleb formations on plasma membrane (arrows). Bar = 10μ.

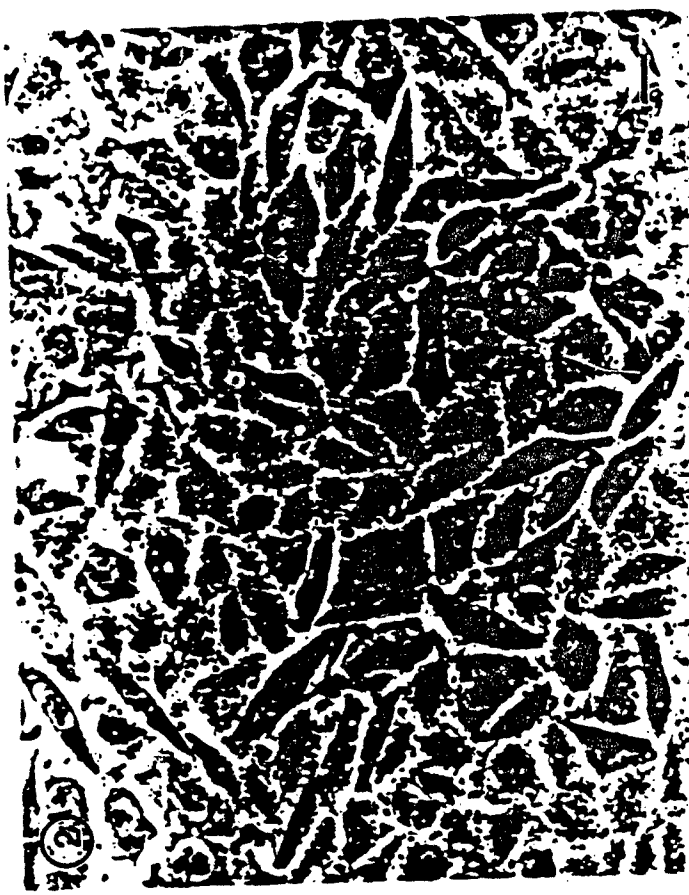
Figure 5. TEM micrographs of control cell cultures (A) CHO, (B) VERO. Note spiral polysomes (box). Bar = 1μ.

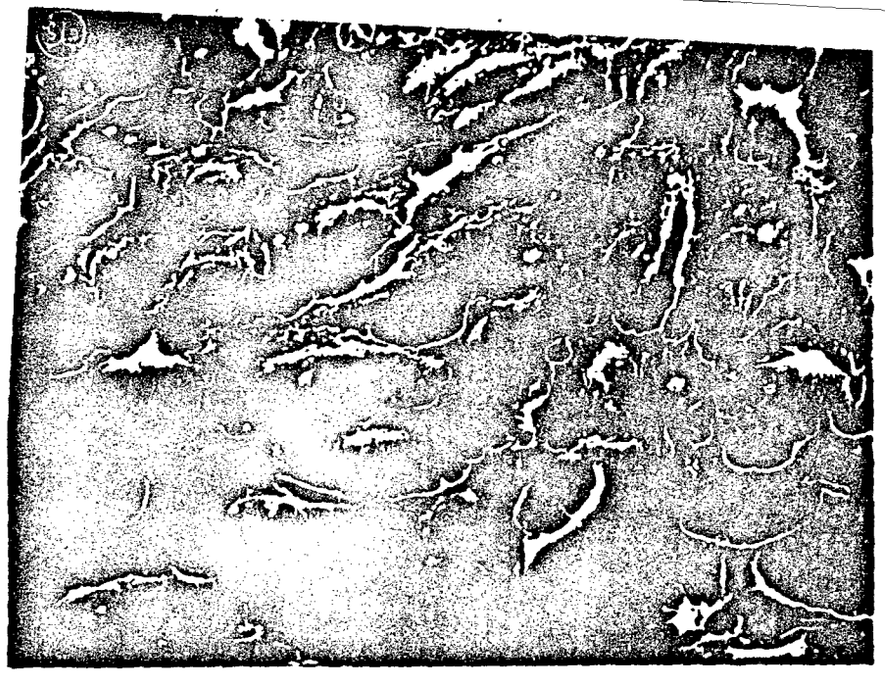
Figure 6. TEM micrograph of CHO cells exposed to 1.0 μg/ml T-2 toxin for 12 hrs. Note membrane bleb formation. Bar = 1μ.

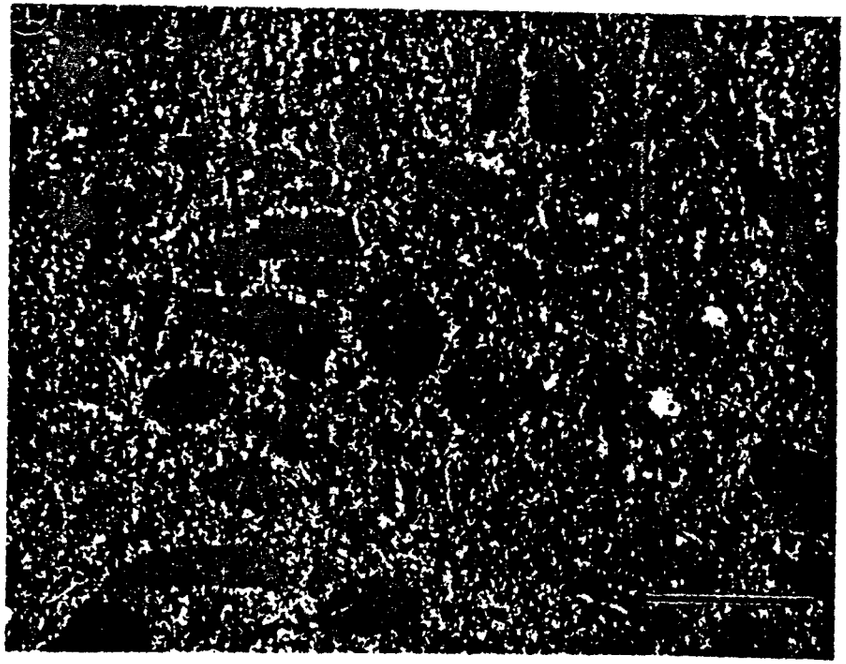
Figure 7. TEM micrographs of cell cultures exposed to 1.0 μg/ml T-2 for 12 hrs. Note monosomes in both cell types. (A) CHO, (B) VERO. Bar = 1μ.

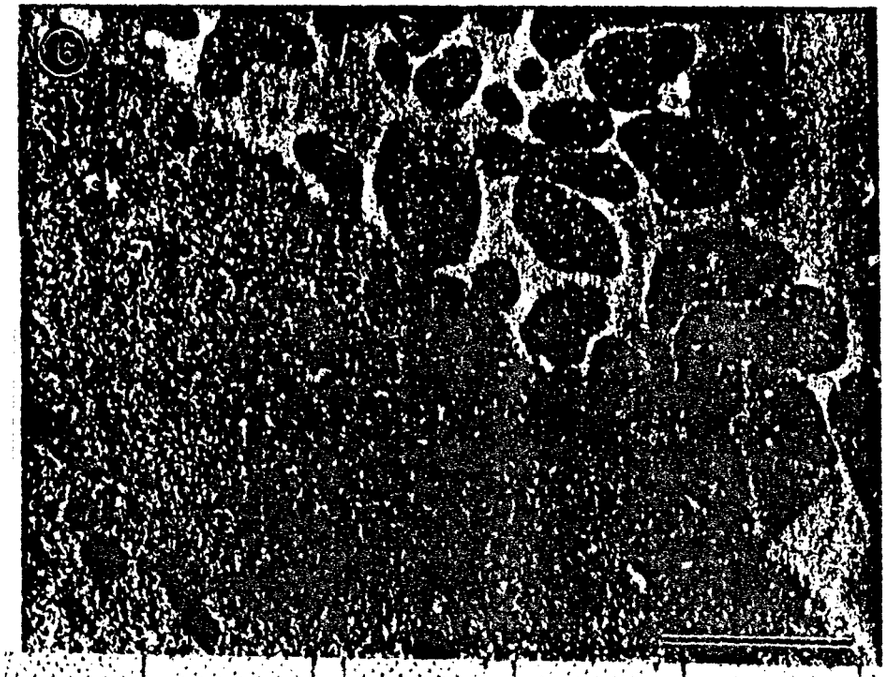
Figure 8. TEM micrograph of CHO cells exposed to 35 μg/ml of puromycin for 12 hrs. Note membrane blebs and dissociated polysomes. Bar = 1μ

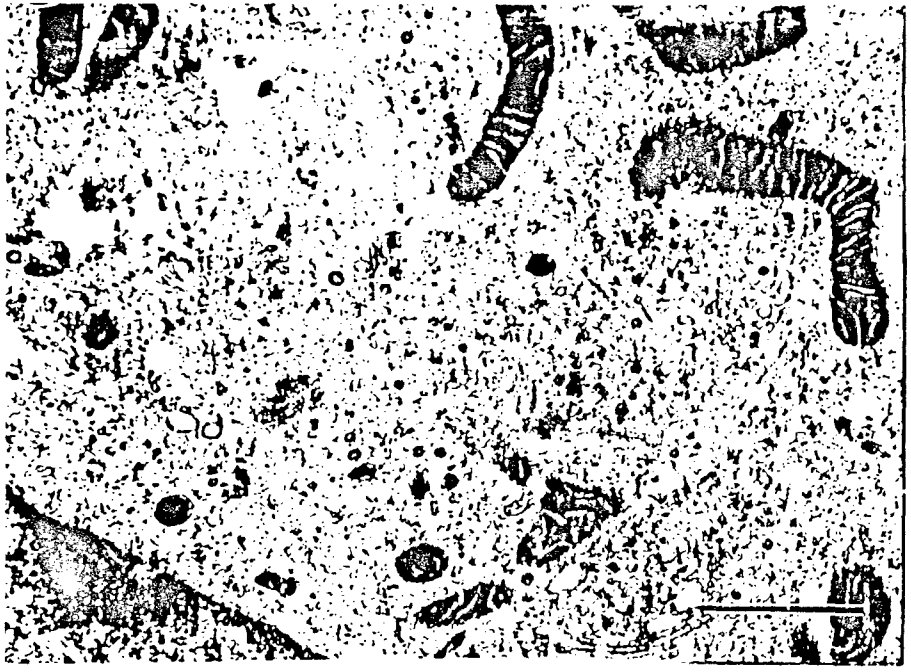
Figure 9. TEM micrograph of VERO cells exposed to 0.01 μg/ml T-2 toxin for 12 hrs. Note bailooned and disorientated cristae formations (arrows). Inset - Note condensed cristae formations in several mitochondria. Bar = 1μ.

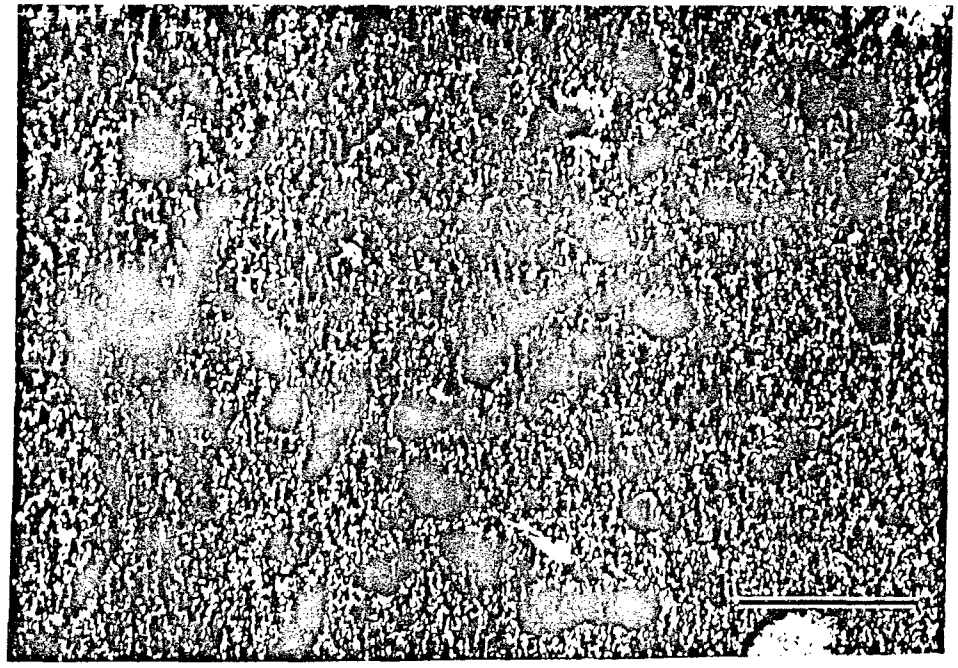


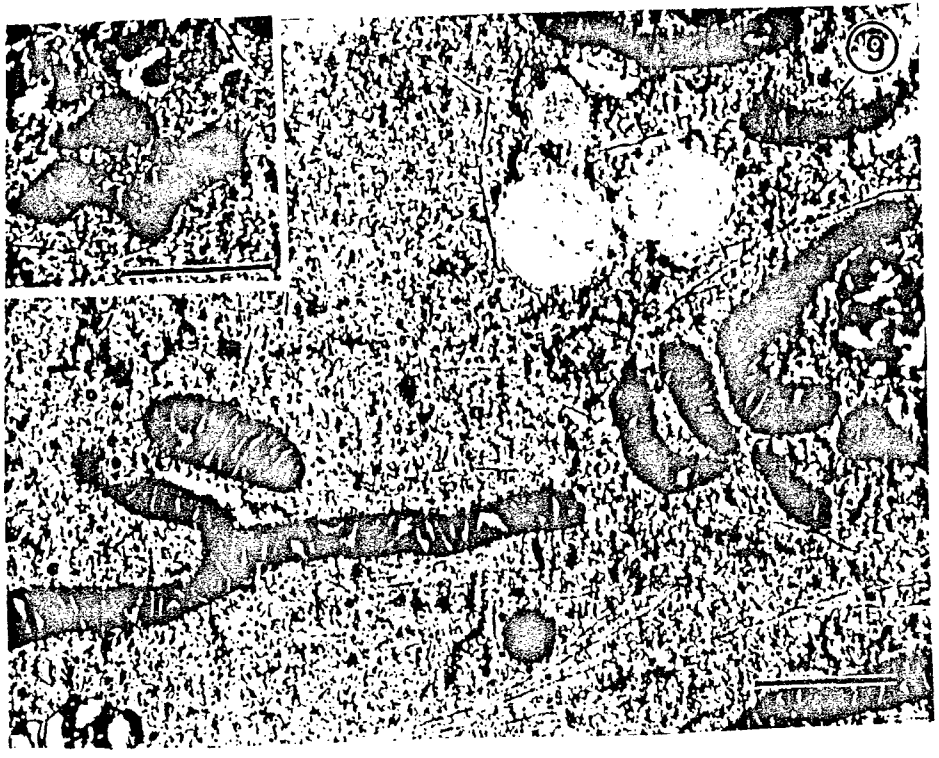








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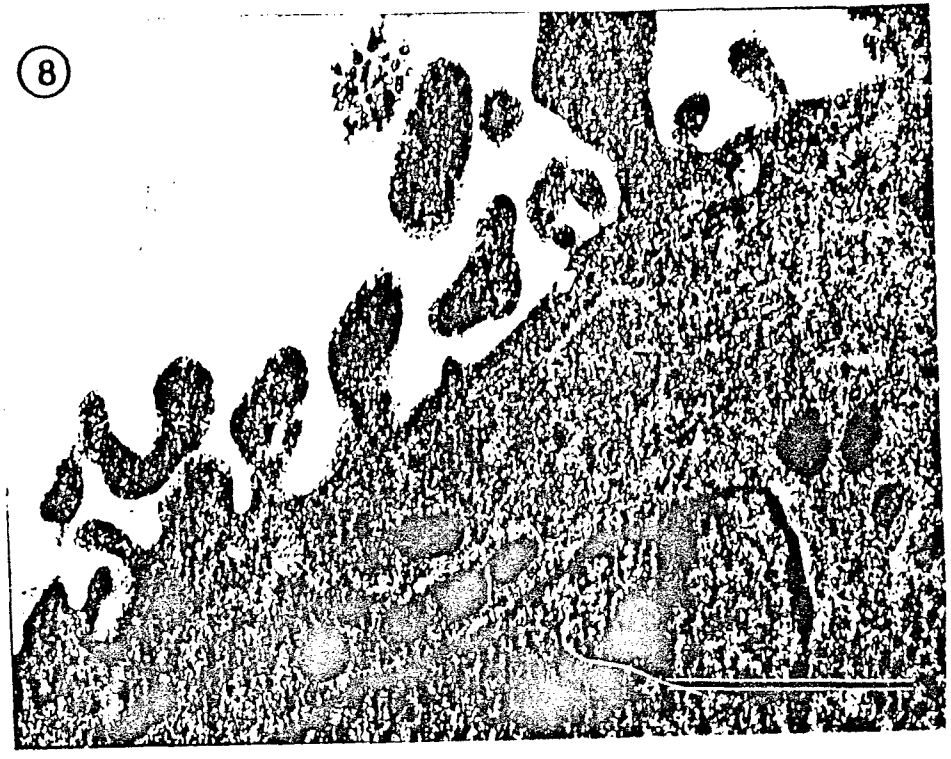
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